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**B2-Lymphocyte responses to oxidative stress-derived antigens contribute to the evolution of nonalcoholic fatty liver disease (NAFLD)**

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# B2-LYMPHOCYTE RESPONSES TO OXIDATIVE STRESS-DERIVED ANTIGENS CONTRIBUTE TO THE EVOLUTION OF NONALCOHOLIC FATTY LIVER DISEASE (NAFLD)

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## Abstract

Recent evidence implicates adaptive immunity as a key player in the mechanisms supporting hepatic inflammation during the progression of experimental nonalcoholic fatty liver disease (NAFLD). In these settings, patients with NAFLD often show an increase in the circulating levels of antibodies against oxidative stress-derived epitopes (OSE). Nonetheless, the actual role of humoral immunity in NAFLD is still unclear. This study investigates the contribution of B-lymphocytes to NAFLD evolution.

B-lymphocyte immunostaining of liver biopsies from NAFLD patients showed that B-cells were evident within cell aggregates rich in T-lymphocytes. In these subjects, B/T-lymphocyte infiltration positively correlated with both circulating IgG targeting oxidative stress-derived epitopes (OSE) and interferon- $\gamma$  (IFN- $\gamma$ ) levels. Furthermore, high prevalence of lymphocyte aggregates identified patients with more severe lobular inflammation and fibrosis. In mouse models of NAFLD, the onset of steatohepatitis was characterized by hepatic B2-lymphocytes maturation to plasma cells and by an elevation in circulating anti-OSE IgG titers. B-cell responses preceded T-cell activation and were accompanied by the up-regulation in the hepatic expression of B-cell Activating Factor (BAFF). Selective B2-cell depletion in mice over-expressing a soluble form of the BAFF/APRIL receptor Transmembrane Activator and Cyclophilin Ligand Interactor (TACI-Ig) prevented plasma cell maturation and Th-1 activation of liver CD4<sup>+</sup> T-lymphocytes. Furthermore, TACI-Ig mice showed milder steatohepatitis and a decreased progression to fibrosis. Similarly, mice treatment with the BAFF-neutralizing monoclonal antibody Sandy-2 prevented hepatic B2-cell responses and ameliorated steatohepatitis.

From these data we conclude that B2-lymphocyte activation is an early event in NAFLD evolution and contributes to the disease progression through the interaction with T-cells. Furthermore, combined clinical and experimental data suggest that elevated circulating anti-OSE IgG can identify a subset of NAFLD patients in whom adaptive immunity has a relevant role in the disease evolution toward fibrosis.

## Introduction

In the last decade, nonalcoholic fatty liver disease (NAFLD) has emerged as a growing cause of liver fibrosis/cirrhosis, while end-stage nonalcoholic steatohepatitis (NASH) is already the second most common indication for liver transplantation in the United States [1]. Moreover, NAFLD/NASH is increasingly recognized as an important cause of hepatocellular carcinoma (HCC) [2]. Beside the effects on the liver, epidemiological data indicate a strong association between NAFLD and the prevalence of extra-hepatic complications, such as type 2 diabetes mellitus, cardiovascular diseases, chronic kidney disease and osteoporosis [3]. In all these settings, hepatic inflammation that characterizes NAFLD evolution to nonalcoholic steatohepatitis (NASH) is considered the driving force for the progression to cirrhosis as well as an independent risk factor for extrahepatic complications [3]. However, the mechanisms responsible for steatohepatitis are still incompletely understood.

Several factors have been proposed to sustain hepatic inflammation in NASH pathogenesis. Among these, the best characterized are persistent parenchymal injury due to oxidative stress and lipotoxicity, as well as inflammasome activation and the direct stimulation of Kupffer cells by either excess of circulating free fatty acids and cholesterol or by gut-derived bacterial products [4-7]. As a result, circulating monocytes are recruited within the liver and, by differentiating into M1 polarized macrophages, further contribute to the release of pro-inflammatory mediators and to oxidative stress-induced damage [8]. Although these mechanisms implicate an extensive involvement of innate immunity, they do not explain the large inter-individual variability in the development of hepatic inflammation.

An emerging body of evidence indicates that, besides the role of innate immune responses, NASH is also characterized by the involvement of adaptive immunity. Indeed, lobular and portal lymphocyte infiltration is a histological feature of human NASH [9], while experimental NASH models show that CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes, B-lymphocytes and natural killer T-cells (NKT) are recruited within the liver in parallel with the worsening of steatohepatitis [10,11]. T-cell subsets in NASH livers express activation markers (CD44, CD69) and show an enhanced production of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-17A, IL-17F and tumor necrosis factor superfamily member 14 (TNFSF14; LIGHT), indicating that lymphocytes infiltrating the liver are functionally activated [10-12]. These findings are supported by clinical observations showing that human NASH is

characterized by an increase in circulating IFN- $\gamma$ -producing CD4<sup>+</sup> T-cells as well as enhanced IFN- $\gamma$  production within the liver [13,14] in relation to CD8<sup>+</sup> T- and NKT-cell infiltration [10,15]. Conversely, the severity of experimental NASH is greatly lowered in Rag1<sup>-/-</sup> mice, which are unable to mount adaptive immune responses [10]. Accordingly, the lack of CD8<sup>+</sup> T- and NKT-cells as well as LIGHT, IFN- $\gamma$  and IL-17 deficiencies ameliorate steatohepatitis and prevent its evolution to HCC [10, 12, 16]. In these settings, we have shown that epitopes derived from oxidative stress (OSE), namely malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE) protein adducts, are involved in the activation of adaptive immunity in NASH [11]. In fact, mice treatment with antioxidants lowers immune responses [17], whereas the immunization with MDA-adducts before NASH induction further stimulates lobular inflammation by promoting Th-1 activation of liver CD4<sup>+</sup> helper T-cells [11]. Besides T-cell-mediated immunity, experimental NASH is also characterized by humoral immune responses involving the production of IgG against OSE [11,17]. Elevated titers of the same antibodies are detectable NAFLD/NASH patients in whom are associated with increased hepatic inflammation [19] and are an independent predictor of advanced fibrosis [18].

These observations and the notion that adipose tissue infiltration by B-lymphocytes plays a key role in supporting systemic inflammation and insulin resistance in obesity [20,21], prompted us to investigate the role of B-lymphocytes in the pathogenesis of NASH.

## **Materials & Methods**

*Human specimen collection and analysis.* Liver biopsies from 41 consecutive patients with NAFLD/NASH, referring to the Liver Unit of the University Hospital of Novara from 2011 to 2016, were analyzed. All samples were collected at the time of first diagnosis. Patients were characterized by anthropometric, clinical and biochemical data and liver biopsies were evaluated for the severity of steatohepatitis and fibrosis according to Kleiner et al. [22]. All subjects gave informed consent to the analysis and the study was planned according to the guidelines of the local ethical committee. The clinical and biochemical features of patients are reported in Supplementary Table 1.

*Animals and experimental protocol.* Transgenic *TACI-Ig* mice on C57BL/6 background [23] were a kind gift of Dr. A. Villunger (Division of Developmental Immunology, Biocenter, Medical University

Innsbruck, Innsbruck, Austria). Eight-week-old male wild type and *TACI-Ig* mice were fed *ad libitum* with either methionine/choline deficient (MCD) diet for 1 or 4 weeks or with a choline deficient and amino acid defined (CDAA) diet for 12 or 24 weeks (Laboratorio Dottori Piccioni, Gessate, Italy). Control animals received the same diets supplemented by either choline/methionine or choline. In some experiments, mice were injected intra-peritoneally with the BAFF neutralizing monoclonal mouse IgG1 Sandy-2 [24] (2 µg/g body weight; Adipogen, Liestal, Switzerland) at the start and after two weeks of MCD diet. Control animals received isotype-matched IgG. The animals were housed at 22°C with alternating 12 hours light/dark cycles. The mice were not fasted before sample collections. In all the experiments euthanasia was performed under isofluorane anesthesia between 9 a.m. and 12 a.m. The experimental procedures complied with the EU guidelines for animal experimentation and were approved by the Italian Ministry of Health.

*Measurement of antibody titers against OSE.* Anti-OSE IgG reactivity was evaluated by enzyme-linked immunosorbent assay (ELISA). Malonyl-dialdehyde (MDA) adducts with either human serum albumin (MDA-HSA; human studies) or bovine serum albumin (MDA-BSA; animal studies) were prepared as previously reported [18] and used to coat polystyrene microwell ELISA plates (Nunc, Roskilde, Denmark).

*Biochemical analysis.* Plasma alanine aminotransferase (ALT) and liver triglycerides were determined by spectrometric kits supplied by Gesan Production S.r.l. (Campobello di Mazara, Italy) and Sigma Diagnostics (Milan, Italy), respectively. Circulating TNF- $\alpha$  was evaluated by commercial ELISA kits supplied by Peprotech (Milano, Italy).

*Histology and immunohistochemistry.* Serial sections from paraffin-embedded human liver biopsies were immune-stained with anti-CD20 and anti-CD3 antibodies (Roche/Ventana, Tucson, AZ, USA) using Bond Polymer Refined Detection kit on the Bond Max auto-stainer (Leika Biosystems, Wetzlar, Germany). The presence of B-/T-cell aggregates was evaluated semi-quantitatively according to the size and number. Hematoxylin/eosin stained mouse liver sections were scored blindly for steatosis, lobular inflammation and fibrosis [11]. Collagen deposition was detected by Picro-Sirius Red staining. Liver activated hepatic stellate cells were evidenced in formalin-fixed sections using an  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) polyclonal antibody (Labvision, Bio-Optica, Milan, Italy) in combination with a horseradish peroxidase polymer kit (Biocare

Medical, Concord, CA, USA). The extension of Sirius Red and  $\alpha$ -SMA-positive areas was quantified by histo-morphometric analysis using the ImageJ software (<https://imagej.nih.gov/ij/>).

*Intrahepatic mononucleated cell isolation and flow cytometry analysis.* Liver mononucleated cells were isolated from the livers of naive and MCD-fed mice and purified on a density gradient (Lympholyte®-M, Cedarlane Laboratoires Ltd. Burlington, Canada) as described in [25]. Cells were washed with Hank's medium and incubated 30 min with de-complemented mouse serum to block nonspecific immunoglobulin binding. The cells were then stained with fluorochrome-conjugated antibodies for CD45, CD3, CD4, CD8, B220, IgM, CD69, CD23, CD43, MHCII CD11c, CD80 (eBiosciences, San Diego CA, USA), CD138 (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) or Attune™ NxT (Thermo Fischer Scientific, Waltham, MS, USA) flow cytometers. Intracellular staining for TNF- $\alpha$ , IFN- $\gamma$  and IL-10 was performed using specific fluorochrome-conjugated antibodies (eBiosciences, San Diego CA, USA) after cell permeabilization with saponin (Permeabilization Kit, eBiosciences, San Diego CA, USA). Single cells were pre-gated on CD45<sup>+</sup>.

*mRNA extraction and Real time PCR.* Liver RNA was retro-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy) in a Tecne TC-312 thermocycler (TecneInc, Burlington NJ, USA). Real Time PCR was performed in a CFX96™ Real-time PCR System (Bio-Rad, Hercules, California, USA) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse TNF- $\alpha$ , IL-12p40, CCL2, CXCL10, IFN- $\gamma$ , CD154, T-bet, BAFF, APRIL,  $\alpha$ 1-procollagen, TGF- $\beta$ 1,  $\alpha$ -SMA and beta-actin (Applied Biosystems Italia, Monza, Italy). All samples were run in duplicate and the relative gene expression was calculated as  $2^{-\Delta Ct}$  over that of  $\beta$ -actin gene. The values were expressed as fold increase over control samples.

*Data analysis and statistical calculations.* Statistical analyses were performed by SPSS statistical software (SPSS Inc. Chicago IL, USA) using one-way ANOVA test with Tukey's correction for multiple comparisons or Kruskal-Wallis test for non-parametric values. Significance was taken at the 5% level. Normality distribution was preliminarily assessed by the Kolmogorov-Smirnov algorithm.

## Results

### *B- and T-lymphocyte responses in human NASH.*

We previously reported that NAFLD/NASH patients have humoral immunity against OSE [18,19]. These findings were confirmed in the present study by measuring circulating IgG targeting MDA-derived adducts in a new cohort of 41 patients (17 male/24 female) with histological diagnosis of NAFLD/NASH. Among these patients, 10 (24%) had steatosis only, 7 (17%) steatosis with mild lobular inflammation, while the remaining 24 (59%) had NASH with variable degrees of fibrosis. As shown in Figure 1A, 18 (43%) of NAFLD/NASH patients had titers of anti-OSE IgG above the control threshold. Furthermore, in agreement with previous observations [18], the prevalence of advanced fibrosis or cirrhosis (staging  $\geq 2$ ) was higher among the subjects with elevated anti-OSE IgG as compared to those with anti-OSE reactivity within the control range (OR=3.25; 95% CI 1.03-15;  $p=0.05$ ). As compared to healthy controls, NAFLD/NASH patients had also elevated circulating IFN- $\gamma$  levels (Fig. 1B). Serum IFN- $\gamma$  was higher among NAFLD/NASH patients showing high anti-OSE IgG reactivity (Fig. 1C) and positively correlated with the severity of fibrosis (Spearman  $r=0.59$ ; 95% CI 0.07-0.86;  $p=0.03$ ). Immunostaining of serial sections from liver biopsies of the same patients using, respectively, the B-cell marker CD20 and the T-cell marker CD3 showed that in 26 (63%) liver specimens CD20<sup>+</sup> B-cells were evident within mononucleated cell aggregates rich of CD3<sup>+</sup> T-lymphocytes (Fig. 1D). The prevalence of B/T-lymphocyte infiltration was independent from age, BMI, HOMA-IR, transaminase release and the degree of steatosis. However, NAFLD/NASH patients with marked/high B-/T-cell infiltration had elevated anti-OSE IgG titers (Fig. 1E) as well as higher scores of lobular inflammation and fibrosis than the subjects with low/mild infiltration (Fig. 1F-G). The number and size of lymphocyte aggregates also positively correlated with circulating IFN- $\gamma$  levels (Spearman  $r=0.45$ ; 95% CI 0.005-0.048;  $p=0.02$ ), lobular inflammation score (Spearman  $r=0.45$ ; 95% CI 0.17-0.67;  $p=0.003$  and  $r=0.39$ ; 95% CI 0.10-0.62;  $p=0.01$ ) and fibrosis staging (Spearman  $r=0.44$ ; 95% CI 0.15-0.66;  $p=0.004$  and  $r=0.41$ ; 95% CI 0.11-0.63;  $p=0.008$ ), suggesting a functional interplay between humoral and cellular immunity in the processes leading to NASH progression.



### *Changes in liver B-lymphocytes during the evolution of experimental NASH*

Based on these observations, we sought to dissect the actual role of liver B-cells in the processes involved in NASH evolution. As obesity has been shown to promote B-cell activation that, on its turn, influences insulin resistance and systemic inflammation [21], for these experiments we relied on obesity-independent models of steatohepatitis based on the administration of a methionine/choline deficient (MCD) or a choline-deficient and amino acid defined (CDAA) diets [26]. These models were also chosen because, according to previous studies [10,11], they reproduce liver lymphocyte responses associated to human NAFLD/NASH. In mice receiving the MCD diet, flow cytometry analysis of liver myeloid cells revealed that the number of IgM<sup>+</sup>/B220<sup>+</sup> hepatic B-lymphocytes significantly declined after one week of treatment (Fig. 2A) in parallel with the onset of steatohepatitis (Supplementary Fig. 1). Such an effect specifically involved the fraction of B220<sup>+</sup>/CD43<sup>-</sup>/CD23<sup>+</sup> B2-lymphocytes, while the pool of B220<sup>+</sup>/CD43<sup>+</sup>/CD23<sup>-</sup> B1-cells was unmodified (Supplementary Fig. 2A-B). Liver B-cell lowering was accompanied by a concomitant up-regulation in the expression of the early lymphocyte activation marker CD69 among B220<sup>+</sup> cells (Supplementary Fig. 2C) and by the expansion of B220<sup>+</sup>/CD138<sup>+</sup> plasma blasts and B220<sup>-</sup>/CD138<sup>+</sup> plasma cells (Fig. 2B). In the same animals, we also detected an increase in the titers of circulating anti-OSE IgG (Fig. 2C), without changes in IgM reactivity against the same antigens (Fig. 2D), indicating B2-cell maturation toward IgG-producing plasma cells. Similar changes in liver B-cell and plasma cell compartments were also observed in mice with steatohepatitis induced by 12 weeks feeding with the CDAA diet (Supplementary Fig. 3), which differently from the MCD diet does not cause weight loss [26]. B-cell responses in NASH were associated to the up-regulation in the liver expression of B-cell Activating Factor (BAFF; TNFSF13b), one of the cytokines regulating B-cell survival and maturation [27] (Supplementary Fig. 2D). No changes were instead observed for the other B-cell regulating cytokine A Proliferation Inducing Ligand (APRIL; TNFSF13) (Supplementary Fig. 2E).

### *B2-lymphocyte deficiency interferes with the onset of immune response in NASH*

It is well known that B-lymphocytes can act as antigen presenting cells for CD4<sup>+</sup> T-cells through the expression of class II Major Histocompatibility Complex (MHCII) and costimulatory molecules [30]. Time-course experiments revealed that an early up-regulation of MHCII among CD138<sup>+</sup> plasma

blasts and plasma cells (Supplementary Fig. 2F-G). Furthermore, B-cell activation preceded the liver recruitment/activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes as well as the up-regulation of liver mRNAs for the Th-1 activation markers IFN- $\gamma$ , T-bet and CD40 ligand (CD154) (Supplementary Fig. 4A & B). To investigate the possible role of B2-lymphocytes in modulating NASH-associated T-cell responses, we took advantage of transgenic mice overexpressing a soluble form of the BAFF/APRIL receptor Transmembrane Activator and Cyclophilin Ligand Interactor (TACI; TNFRSF13B) fused with the Fc portion of human IgG1 (*TACI-Ig* mice) [23]. These mice are characterized by an impaired B-cell maturation in the periphery, leading to a severe depletion of marginal zone and follicular B2-lymphocytes, but not of peritoneal B1-cells [23]. In our hands, *TACI-Ig* mice showed a marked lowering of hepatic B-cells specifically involving the B220<sup>+</sup>/CD43<sup>-</sup>/CD23<sup>+</sup> B2-subset (Supplementary Fig. 5A). Conversely, no significant changes were appreciable in the fraction of B220<sup>+</sup>/CD43<sup>+</sup>/CD23<sup>-</sup> B1-cells (Supplementary Fig. 5B). Upon the induction of NASH, we observed that liver plasma cell maturation, as well as the production of anti-OSE IgG, were impaired in *TACI-Ig* mice as compared to wild-type littermates (Fig. 3A-B). In line with a role of B2-cells in promoting T-cell responses, the liver recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes, as well as their CD69 expression, were significantly reduced in *TACI-Ig* mice receiving the MCD diet (Fig. 3C-D), in spite the expansion and activation of hepatic dendritic cells were not affected (Supplementary Fig. 5C). Moreover, the hepatic expression of Th-1 activation markers IFN- $\gamma$ , CD154 and T-bet was also decreased in *TACI-Ig* mice (Fig. 3E). These effects were specific for liver immune responses associated with NASH, as no difference in the development of anti-OSE immunity were evident between wild-type and *TACI-Ig* mice following immunization with bovine serum albumin complexed with malonyl-dialdehyde (Supplementary Fig. 6). The lack of B2-cells in *TACI-Ig* mice also appreciably ameliorated lobular inflammation score ( $2.3 \pm 0.5$  vs  $1.6 \pm 0.5$ ;  $p < 0.05$ ) and the prevalence of necrotic foci ( $7.3 \pm 3.31$  vs  $4.0 \pm 1.3$ ;  $p < 0.05$ ) without affecting the extension of steatosis ( $2.8 \pm 0.4$  vs  $2.5 \pm 0.5$ ;  $p = 0.39$ ).

#### *Effects of BAFF neutralization ameliorates the evolution of NASH*

Previous studies have shown that circulating levels of BAFF are higher in patients with NASH than in those with simple steatosis, and correlate with the severity of steatohepatitis and fibrosis [28]. Thus, to investigate the effects of interfering with BAFF on the evolution of steatohepatitis, we

performed additional experiments taking advantage of the BAFF neutralizing monoclonal antibody Sandy-2 [24]. In preliminary tests we observed that treatment for one week with Sandy-2 (2 µg/g body weight) reduced by about 40% circulating and liver B-cells, specifically affecting the B2-subset (Supplementary Fig. 7A-B). Accordingly, the administration of Sandy-2 prevented liver plasma cell maturation in mice fed with the MCD diet for 1 week (Supplementary Fig. 7C). In the animals receiving the MCD diet for 4 weeks, BAFF neutralization ameliorated histological scores for steatosis ( $2.8 \pm 0.4$  vs  $1.7 \pm 0.8$ ;  $p < 0.05$ ) and lobular inflammation ( $2.7 \pm 0.5$  vs  $1.8 \pm 0.4$ ;  $p < 0.05$ ) as well as ALT release and liver triglycerides (Fig. 4A-C). Differently from what observed in TACI-Ig mice, Sandy-2 treatment did not appreciably affect the prevalence of liver infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (not shown). Nonetheless, Th-1 activation of liver CD4<sup>+</sup> T-lymphocytes, as evaluated by IFN-γ production, was significantly lowered by Sandy-2 treatment (Fig. 4D). BAFF blockage also decreased the hepatic expression of pro-inflammatory mediators such as TNF-α, IL-12 and CXCL10 (Fig. 4E).

#### *Role of B-lymphocyte in NASH progression to fibrosis*

Besides the improvement of hepatic inflammation, MCD-fed mice receiving Sandy-2 showed a descending trend in the expression of pro-fibrotic markers α1-procollagen and α-smooth muscle actin (α-SMA) (Fig. 4F), although the differences did not reach statistical significance. Based on these results, we investigated whether the lack of B2-lymphocytes would affect the fibrogenic evolution of NASH in *TACI-Ig* mice fed 24 weeks with CDAA diet. Such an experimental model allows, in fact to reproduce well defined hepatic fibrosis avoiding the severe malnutrition that characterizes advanced NASH in mice fed with the MCD diet [26]. In this setting, we observed that *TACI-Ig* mice had hepatic expression of α1-procollagen, α-SMA and Transforming Growth Factor-β1 (TGF-β1) significantly lower than wild-type littermates (Fig. 5A). Consistently, Sirius Red staining for collagen and the prevalence of α-SMA-positive activated hepatic stellate cells were also significantly reduced in *TACI-Ig* mice (Fig. 5B-D). The improvement of fibrosis observed in CDAA-fed *TACI-Ig* mice was associated with a lowering of transaminases release and lobular inflammation (Supplementary Fig. 8), supporting the importance of B-cells in the mechanisms leading to steatohepatitis progression.

## Discussion

Recent observations implicate B-lymphocytes as important players in supporting insulin resistance and systemic inflammation in obesity [20, 21]. In more detail, B-cells isolated from visceral fat of obese mice show an increased production of pro-inflammatory cytokines, while their accumulation in the adipose tissue associates with T-cell and macrophage activation [29, 30]. Moreover, fat inflammation and insulin resistance are lowered in mice lacking B-cells or following B-cell depletion using anti-CD20 antibodies [30]. Recently, Zhang and co-workers have reported that feeding a high fat diet to mice leads to the liver recruitment of cells from B lineage (B220<sup>+</sup>) producing pro-inflammatory cytokines [31]. However, this work did not investigate the actual role of B-cells in the pathogenesis of steatohepatitis. Our present data address this aspect by showing that B-cell activation is an early event in the evolution of experimental NASH and, even in the absence of obesity, contributes in supporting immune responses associated with the progression of steatohepatitis.

B-cells represent about 50% of intrahepatic lymphocytes. However, so far conflicting results have been obtained in studies investigating their role in liver diseases [32-34]. In mice, the pool of liver B-lymphocytes mainly consists of bone marrow-derived mature B220<sup>+</sup>/IgM<sup>+</sup>/CD23<sup>+</sup>/CD43<sup>-</sup> B2-cells resembling spleen follicular B-cells [32]. We have observed that B-cell changes in NASH specifically involve the B2 compartment and are characterized by their maturation to plasma cells. The circulating levels of IgG targeting OSE also increase at the onset of experimental NASH, suggesting that oxidative damage associated with the development of steatohepatitis leads to the generation of antigens recognized by B-cells that then undergo maturation to IgG-producing plasma cells.

The actual relevance of B-cell responses in the pathogenesis of NASH is supported by experiments using B2-cell-deficient *TACI-Ig* mice or by using the BAFF neutralizing antibody Sandy-2. In these settings, B2-cell depletion or the interference with BAFF-mediated survival and maturation of B2-cells ameliorates both parenchymal damage and lobular inflammation and reduces the development of fibrosis. Interestingly B-cell maturation to plasma cells has been recently shown to foster HCC development in mice with steatohepatitis [35]. The beneficial effects connected with the interference with B-cell survival/maturation likely depend upon the reduction in the production of pro-inflammatory mediator by B-lymphocytes [36] as well as the impairment of their

antigen presenting capabilities [37]. On this latter respect, we have observed that B-cell activation in NASH associates with the up-regulation in MHCII molecules in plasma blasts and precedes the liver recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes, while interfering with B2-cells reduces Th-1 activation of CD4<sup>+</sup> T cells without affecting the maturation of dendritic cell. Altogether these results suggest that B2-lymphocytes can have a role in promoting OSE presentation to T-cells that, in turn, support NASH progression [10,11]. Indeed, B-cells express a variety of receptors that can recognize OSE [38], while Béland and co-workers [34] have reported that B-cell depletion with anti-CD20 antibodies ameliorates experimental autoimmune hepatitis by preventing autoantigen presentation to CD4<sup>+</sup> and CD8<sup>+</sup> T-cells.

It is noteworthy that the above mechanisms have several analogies with that observed in atherosclerosis, where B2-lymphocytes activation by OSE represents a key mechanism in plaque evolution. In fact, elevated circulating titers of anti-OSE IgG associate with an enhanced risk of atherosclerosis complications in humans [39], whereas the interference with B2-cells or BAFF-mediated signals reduces experimental atherosclerosis [39,40]. Although NAFLD/NASH is increasingly recognized as an independent risk factor for cardiovascular diseases [3], it is still unclear whether such association might be related to the common involvement of OSE-mediated immune responses. The studies on atherosclerosis have also outlined a dual role of B-cells in the disease evolution. In one hand, in fact, B2-cells promote plaque evolution by supporting humoral and cellular anti-OSE immunity, while the activation of the B1 subset exerts a protective action mainly through the production of natural antibodies capable of scavenging pro-atherogenic oxidized low density lipoproteins (LDL) [39,40]. Natural antibodies are pre-existing germline encoded antibodies belonging to the IgM class with a broad specificity to pathogens, but also able to cross-react with endogenous antigens, such as OSE in oxidized LDL [39]. In line with these notions, studies by Binder's group have shown that B1-lymphocytes can affect the severity of steatohepatitis in LDL receptor-deficient mice fed with high fat/cholesterol diet [41,42]. Such effects have been ascribed to the capacity of B1-lymphocytes to produce IgM cross-reacting with OSE that prevent oxidized LDL accumulation within liver macrophages and their pro-inflammatory activation due to cholesterol engulfment [41]. In our hands, liver B1-cells, as well as circulating IgM, are not modified during NASH evolution, while TACI over-expression or Sandy-2 treatment do not affect the B1-compartment. Nonetheless, the combination of our present data and those by Binder's group suggests the possibility that B1- and B2-cells might have antagonist activities also in

the pathogenesis of NAFLD/NASH, with B2-lymphocytes being involved in promoting pro-inflammatory mechanisms and the B1 subset possibly exerting a protective action. Opposite B1/B2 actions have also recently been documented in relation to B-lymphocyte involvement in promoting obesity-associated adipose tissue inflammation and insulin resistance [43,44]. Such a duality will need to be considered in future studies addressing the role of B-cells in NAFLD, also in relation to the fact that both subsets can give rise to regulatory B-lymphocytes [45].

Although the interference with cytotoxic and inflammatory mechanisms might account for the improvement in liver fibrosis observed in B2-cell-deficient *TACI-Ig* mice with NASH, we cannot exclude that additional mechanisms might also be involved. In fact, previous studies indicate that B-cells can directly contribute to liver fibrogenesis [32,46] through the production of pro-inflammatory mediators that stimulate hepatic stellate cell (HSC) and liver macrophage activation [46]. On the other hand, Thapa and co-workers have reported that during chronic liver injury activated HSCs can support liver B-cell survival and maturation to plasma cells by secreting retinoic acid [46], thus suggesting a complex interplay between B-cells and other non-parenchymal cells in the evolution of chronic liver diseases.

The capacity of B-cells to stimulate inflammation and fibrogenesis through multiple interactions with T-lymphocytes and HSCs accounts for our clinical observations, showing that the prevalence of B-/T-lymphocyte aggregates in liver biopsies of NAFLD/NASH patients correlates with more severe lobular inflammation and enhanced fibrosis. In these subjects, intra-hepatic B-/T-cell aggregates are also associated with elevated titers of anti-OSE IgG and high IFN- $\gamma$  circulating levels, further supporting the clinical relevance of the interplay between B- and T-cells in the processes leading to NAFLD/NASH progression.

## Conclusions

Altogether these data indicate that B2-lymphocyte activation in response to OSE is an early event in NASH evolution and contributes to sustain hepatic inflammation through the interaction with T-cells. These observations, along with previous data indicating anti-OSE IgG as an independent risk factor for NASH progression to advanced fibrosis [18], suggest that the measure of circulating anti-OSE IgG can identify a sub-set of NAFLD/NASH patients in whom adaptive immunity triggered by

oxidative stress might have a relevant role in promoting steatohepatitis and the disease evolution toward fibrosis. If confirmed in prospective studies, IgG reactivity toward OSE might become a useful non-invasive marker for the identification of this sub-set of NAFLD patients who might benefit from already available treatments that interfere with B-cell functions.

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### **Abbreviations**

BAFF: B-cell Activating Factor

CDAA: choline-deficient and amino acid defined

IFN- $\gamma$ : interferon- $\gamma$

NAFLD: nonalcoholic fatty liver disease

NASH: nonalcoholic steatohepatitis

MHCII: class II Major Histocompatibility Complex

MCD: methionine-choline deficient

MDA: malonyl-dialdehyde

OSE: oxidative stress-derived epitopes

$\alpha$ -SMA:  $\alpha$ -smooth muscle actin

TACI: Transmembrane Activator and Cyclophilin Ligand Interactor

TGF- $\beta$ 1: Transforming Growth Factor- $\beta$ 1

TNF- $\alpha$ : Tumor Necrosis Factor- $\alpha$

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## Figure legends

Figure 1: B- and T-lymphocyte responses associates with the evolution of human NAFLD/NASH. (A-C) IgG reactivity toward OSE was measured in 41 NAFLD/NASH patients by in house ELISA assay using as antigen malonyl-dialdehyde (MDA) adducts with human serum albumin. Circulating interferon- $\gamma$  (IFN- $\gamma$ ) was measured in 34 of the patients. For comparison 32 and 18 age/gender matched healthy subjects were used as controls. (D) Immuno-histochemical detection of cell aggregates containing CD20<sup>+</sup> B-lymphocytes and CD3<sup>+</sup> T-lymphocytes in serial sections from liver biopsies of two different representative NAFLD/NASH patients (Magnification 400x). (D-F) The prevalence of B/T-cells aggregates was associated with anti-OSE IgG titers as well as with the severity of lobular inflammation and hepatic fibrosis as estimated according to Kleiner et al. (22). Lymphocyte infiltration was evaluated semi-quantitatively taking into account the number and size of lymphocyte aggregates.

Figure 2: B-cell responses during the evolution of experimental NASH.

Wild type mice were fed with the MCD diet for either 1 or 4 weeks. The intrahepatic distribution of B-lymphocytes, plasma blasts and plasma cells were evaluated by flow cytometry in parallel with the production of IgG targeting OSE and the liver expression of BAFF. (A) Changes in the liver distribution of total IgM<sup>+</sup>/B220<sup>+</sup> B-lymphocytes at different stages of NASH evolution. (B) Changes in the liver distribution of B220<sup>+</sup>/CD138<sup>+</sup> plasma blasts and B220<sup>-</sup>/CD138<sup>+</sup> plasma cells at different stages of NASH evolution. The values are means  $\pm$  SD of three different experiments with 3-4 animals per group. (C-D) changes in anti-OSE antibody titres, as measured by IgG and IgM targeting malonyl-dialdehyde (MDA) adducts in the sera of mice with NASH. The values in panels C and D are means  $\pm$  SD of 8-10 animals per group and the boxes include the values within 25<sup>th</sup> and 75<sup>th</sup> percentile, while the horizontal bars represent the median. The extremities of the vertical bars (10<sup>th</sup>-90<sup>th</sup> percentile) include 80% of the values.

Figure 3: B2-lymphocyte deficiency interferes with the onset of immune responses in NASH.

B2-cell deficient *TACI-Ig* and wild type mice were fed with control or MCD diet for 4 weeks. The intrahepatic distribution of T-lymphocytes and plasma cells was evaluated by flow cytometry in parallel with the production of IgG targeting OSE and the liver expression of Th-1 activation markers. (A) Effect of B2-cell depletion on the liver expansion of B220<sup>-</sup>/CD138<sup>+</sup> plasma cells and (B) the increase of circulating anti-OSE antibody titres, as measured by IgG targeting malonyl-dialdehyde (MDA) adducts. (C) The liver distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes and (D) their expression of the activation marker CD69 in *TACI-Ig* and wild-type mice with NASH. The values in panels A-C are means  $\pm$  SD of three different experiments with 3-4 animals for each group. (E) Down-modulation in the expression of Th-1 activation markers interferon- $\gamma$  (IFN- $\gamma$ ), T-bet and CD40 ligand (CD154) in the liver of *TACI-Ig* mice. The values of RT-PCR analysis are expressed as fold increase over their relative controls and are means  $\pm$  SD of 8-10 animals per group. The boxes in panels B and E include the values within 25<sup>th</sup> and 75<sup>th</sup> percentile, while the horizontal bars

represent the median. The extremities of the vertical bars (10<sup>th</sup>-90<sup>th</sup> percentile) include 80% of the values.

Figure 4: Mice treatment with the BAFF-neutralizing antibody Sandy-2 ameliorates steatohepatitis.

Wild type mice were injected with Sandy-2 mAb (2 µg/g body weight) or isotype-matched IgG at the start and after 2 weeks of MCD diet. (A) Haematoxylin/eosin staining of liver sections (Magnification 200x). (B) Alanine aminotransferase (ALT) release and (C) liver triglyceride content. (D) Flow cytometry evaluation of interferon-γ (IFN-γ) production by CD3<sup>+</sup>/CD4<sup>+</sup> helper T-lymphocytes. The values are means ±SD of three different experiments with 4 animals per group. The hepatic mRNA levels of (E) pro-inflammatory mediators TNF-α, IL-12p40 and CXCL10 and (D) fibrosis markers α1-procollagen and α-smooth muscle actin (α-SMA). RT-PCR values are expressed as fold increase over control values after normalization to the β-actin gene. The values in the panels B, C, E and F refer to 6-7 animals per group and the boxes include the values within 25<sup>th</sup> and 75<sup>th</sup> percentile, while the horizontal bars represent the median. The extremities of the vertical bars (10<sup>th</sup>-90<sup>th</sup> percentile) include 80% of the values.

Figure 5: B2-lymphocyte deficiency reduces NASH evolution to fibrosis.

*TACI-Ig* and wild type (WT) mice were fed with either control or CDAA diets for 24 weeks. (A) Hepatic expression of fibrogenesis markers α1-procollagen, α-smooth muscle actin (α-SMA) and Transforming Growth Factor-β1 (TGF-β1). RT-PCR values are expressed as fold increase over control values after normalization to the β-actin gene. (B) Collagen deposition as detected by Sirius Red staining in representative liver sections from 24-week CDAA diet in WT and *TACI-Ig* mice (Magnification 200x). (C) Immuno-histochemical staining for α-SMA-positive hepatic stellate cells (HSCs) liver sections from 24-week CDAA diet in WT and *TACI-Ig* mice (Magnification 200x). (D) Histo-morphometric analysis of Sirius Red and α-SMA positive areas. The values refer to 5-7 animals per group and the boxes include the values within 25<sup>th</sup> and 75<sup>th</sup> percentile, while the horizontal bars represent the median. The extremities of the vertical bars (10<sup>th</sup>-90<sup>th</sup> percentile) include 80% of the values.

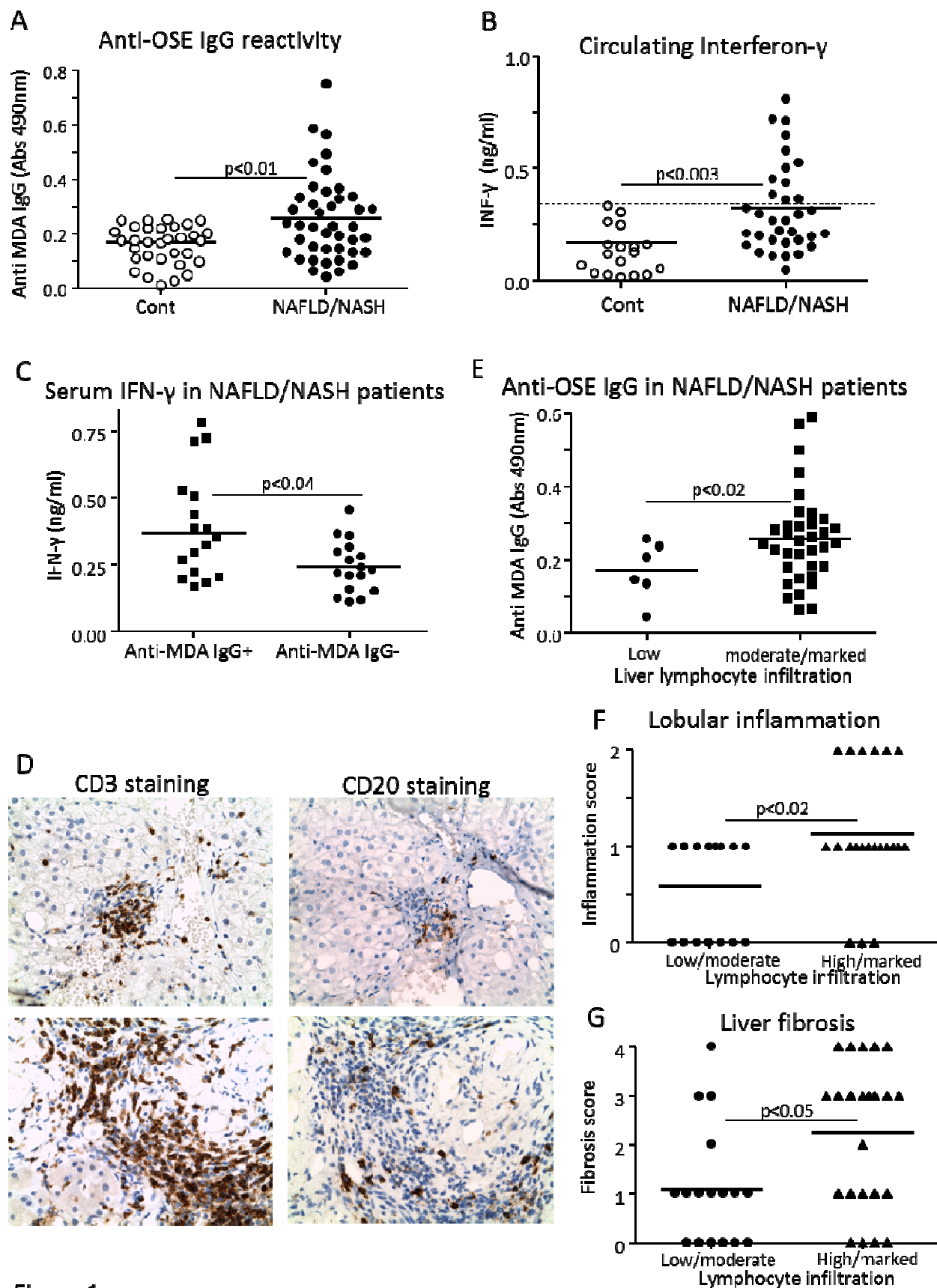


Figure 1

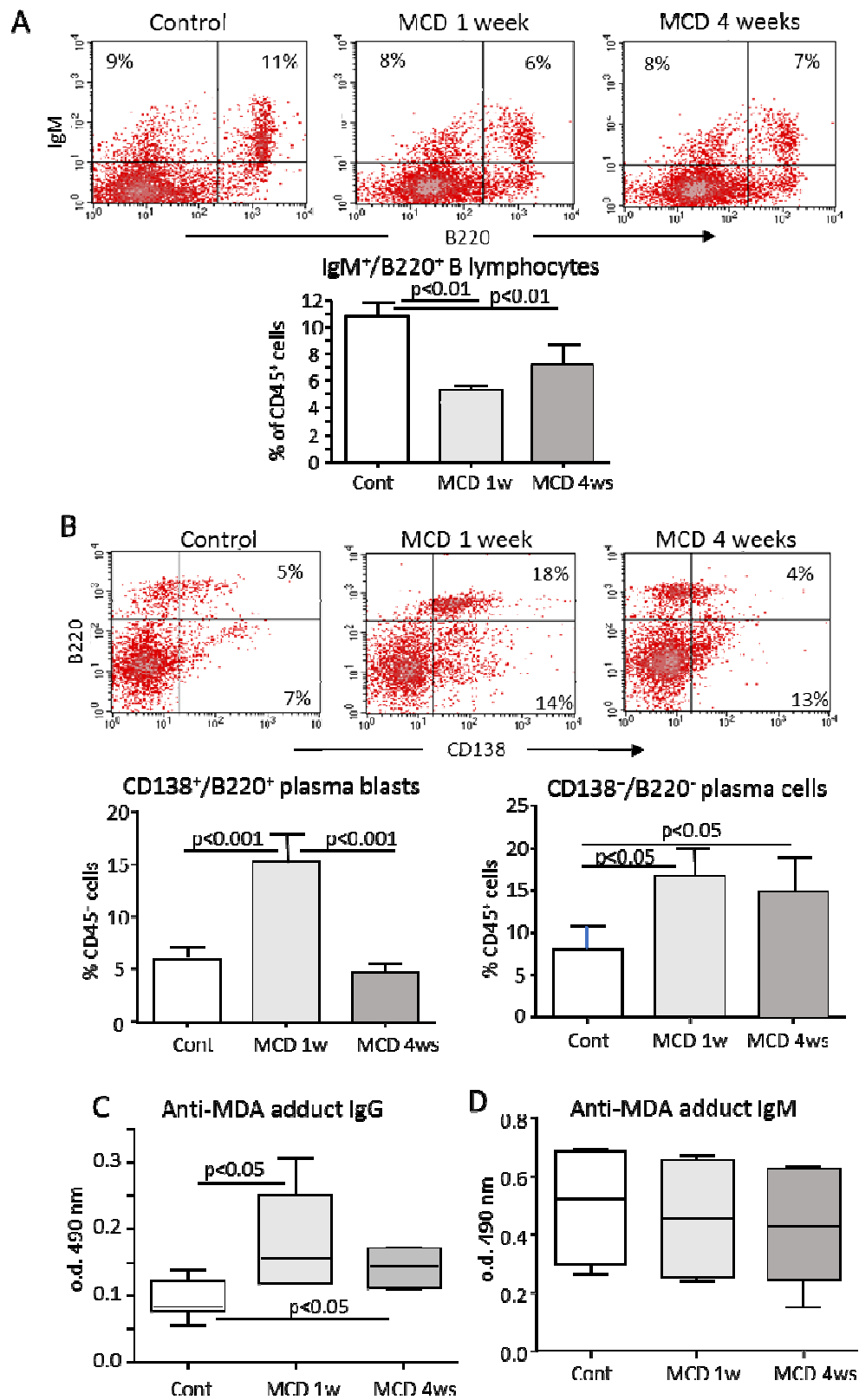


Figure 2

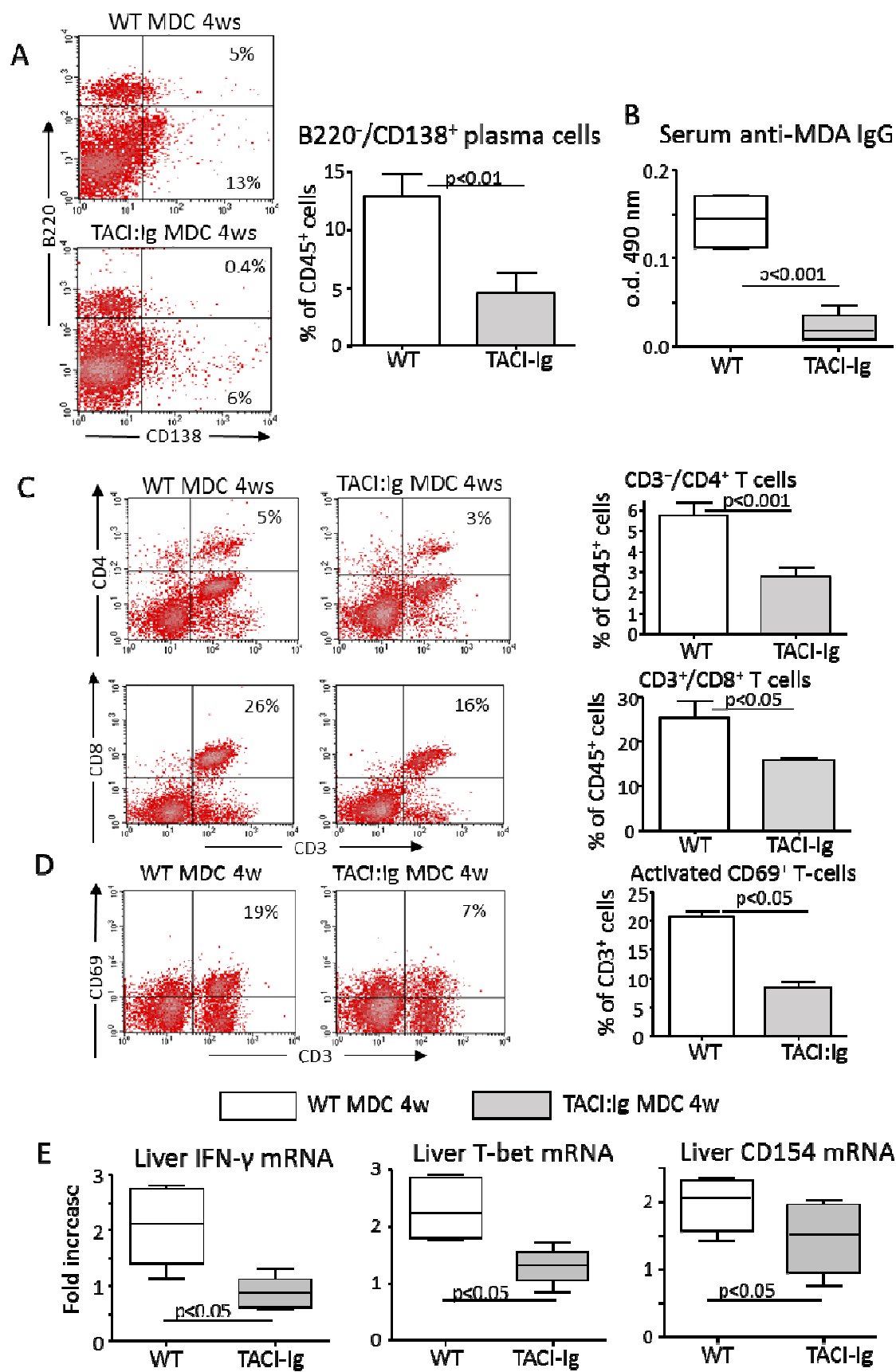


Figure 3



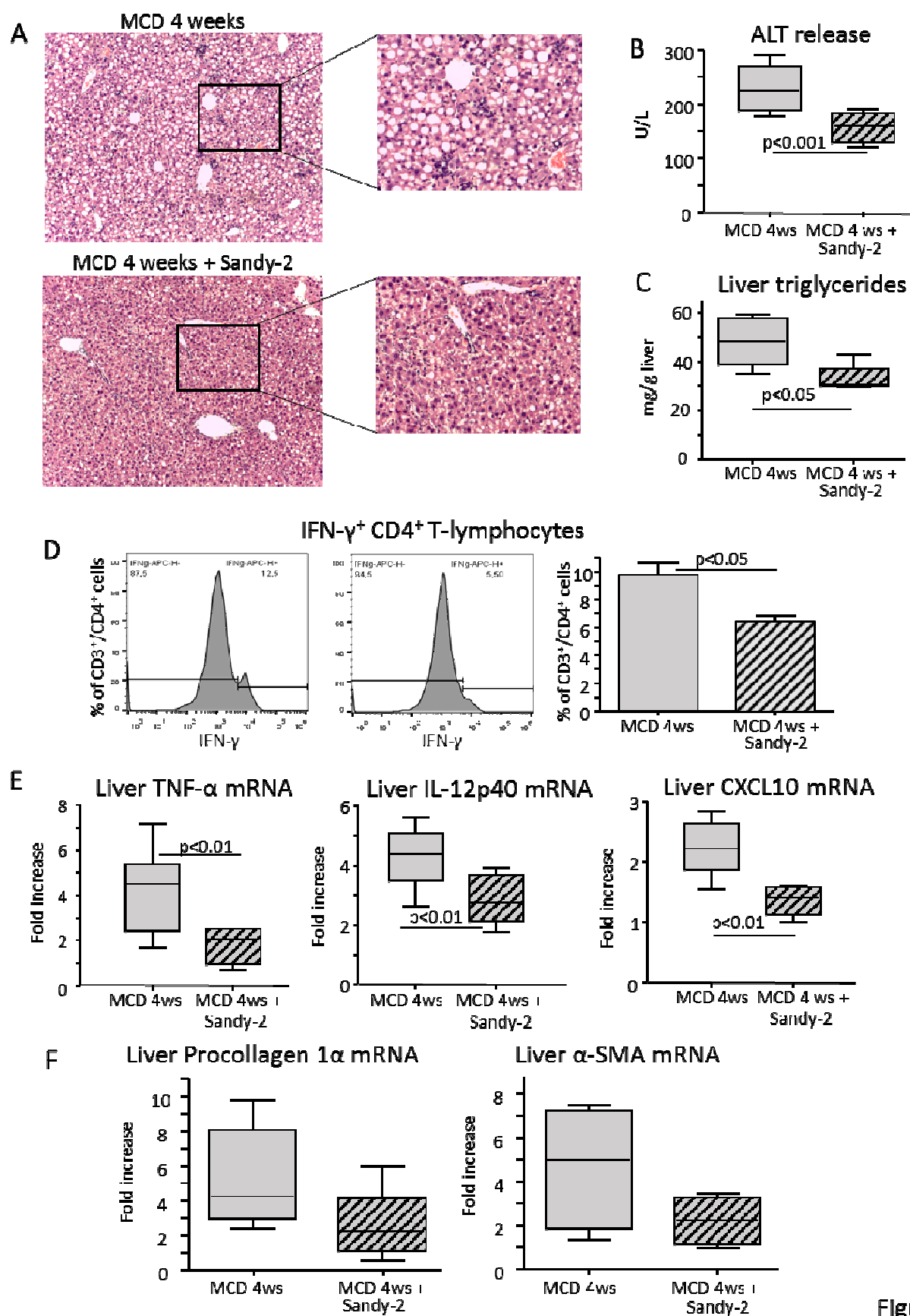


Figure 4

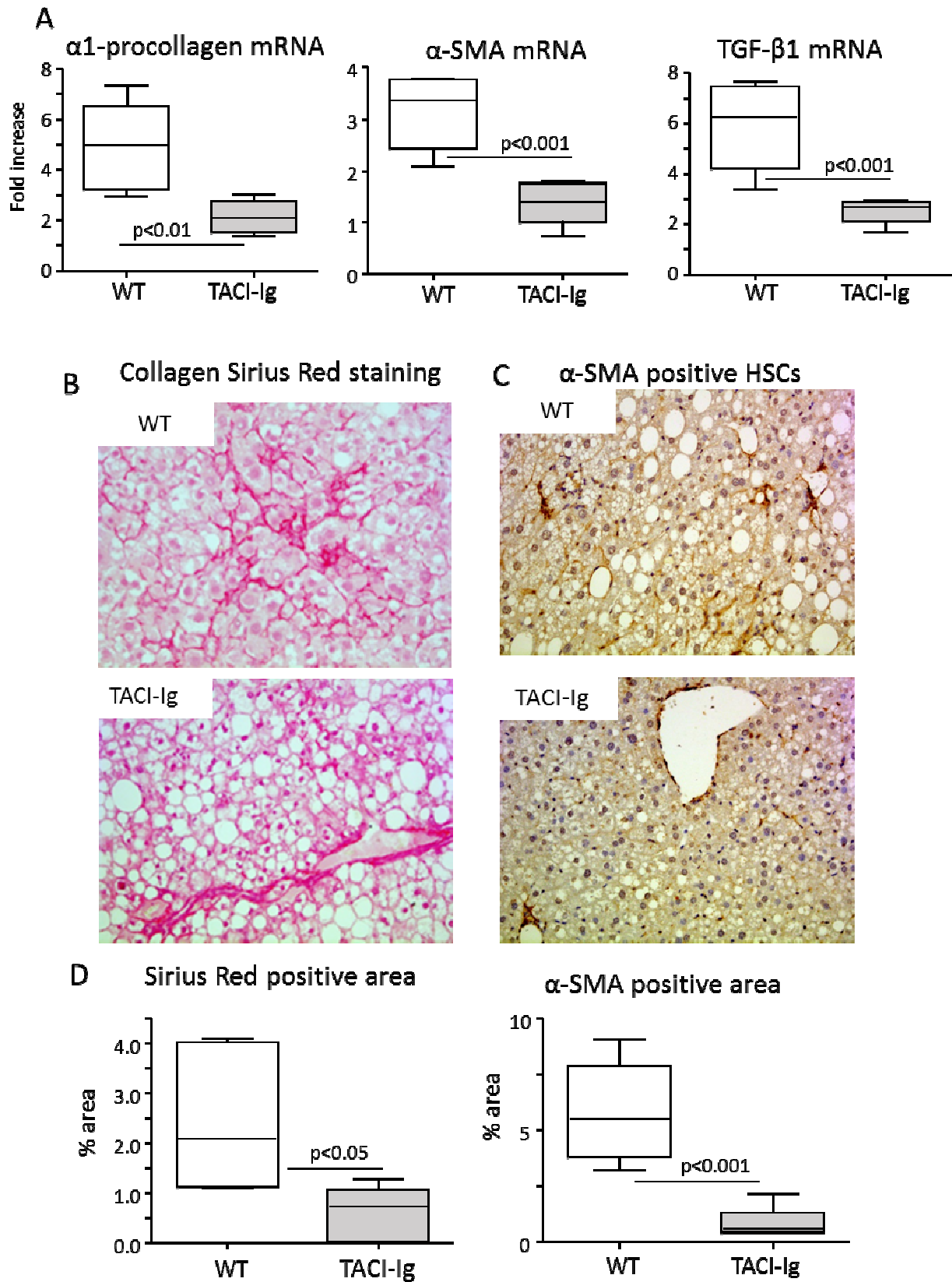


Figure 5

## List of supplementary material

Supplementary Table 1. Clinical and biochemical characterization of NAFLD patients investigated.

Supplementary Figure 1: Time dependent variations of hepatic injury and inflammation during the evolution of NASH induced by mice feeding with a methionine/choline deficient (MCD) diet.

Supplementary Figure 2: B-cell responses during the evolution of experimental NASH specifically involve the B2 sub-set.

Supplementary Figure 3: B-cell responses in experimental NASH induced by choline-deficient amino acid defined (CDAA) diet.

Supplementary Figure 4: Time course of T-lymphocyte recruitment and activation during the evolution of experimental NASH.

Supplementary Figure 5: Characterization of hepatic B-cells and dendritic cells in *TACI-Ig* mice.

Supplementary Figure 6: B2-cell deficiency in *TACI-Ig* mice does not affect the development of anti-OSE immunity.

Supplementary Figure 7: Effects of BAFF-neutralizing monoclonal antibody Sandy-2 on circulating and hepatic B cells.

Supplementary Figure 8: B2-cell deficiency in *TACI-Ig* mice ameliorates experimental NASH induced by choline-deficient amino acid defined (CDAA) diet.